

Detection of *Ascaris suum* in the Livers of Chickens Infected Naturally by the Nested Multiplex PCR Assay

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Abstract

This study was conducted to detect *Ascaris suum*, *Toxocara canis*, and *Toxocara cati* in naturally infected chicken livers, and then to provide information related to ascarid infections in humans. Ninety-four chicken liver samples collected at a fresh market were used for this study. DNA was extracted from each minced liver sample (500 mg) by the alkaline lysis method using NaOH 50mM and Tris-HCl (pH 8.0). The nested multiplex PCR assay using ascarid universal primers was applied to amplify the ITS1 ribosomal RNA gene of *A. suum*, *Toxocara canis*, and *Toxocara cati* in all the liver samples. Then, species specific-primers were used to discriminate between *A. suum* and *Toxocara* spp. The results showed that 2 of the 94 chicken liver samples detected positive for the presence of *A. suum* DNA. This study provided useful information and evidence about *A. suum* infection in humans via exposure to contaminated soil or eating raw/undercooked chicken livers in Vietnam.

Keywords

Ascaris suum, chicken liver, nested multiplex PCR, white spot lesions

Introduction

Acaris suum, along with *Toxocara canis* and *Toxocara cati*, are common roundworms in pig, dog, and cat intestines, and are considered as the causal agents of ascarid larva migrans syndrome (ascarid LMS) in humans. These roundworms have a direct life cycle in which the process of egg embryonation occurs outside in the environment, and they do not need any intermediate hosts to be at an infective stage. Once definitive hosts ingest ascarid embryonated eggs, larvae emerge in the digestive system, penetrate the intestinal wall, and then migrate to the liver via the mesenterial blood veins.

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From the liver, larvae are carried to the lungs using the efferent bloodstream, penetrate the lung alveoli, and then migrate up the respiratory tree. Finally, larvae are coughed up and swallowed by the host to reach the small intestine where they develop into adult worms. The prepatent period is about 6-8 weeks.

Toxocariasis caused by *T. canis* and *T. cati* was first recorded in the 1950s, followed by *A. suum* infection, and impacts many countries around the world (Beaver *et al.*, 1952; Thompson *et al.*, 1986; Chomel *et al.*, 1993; Magnaval *et al.*, 1993; Maruyama *et al.*, 1996; Sakakibara *et al.*, 2002; Hoenigl *et al.*, 2010; Izumikawa *et al.*, 2011; Penelli *et al.*, 2011; Miller *et al.*, 2015). In Vietnam, some researchers have reported toxocariasis in humans (Tran *et al.*, 2014; Do *et al.*, 2016), however, there has been no information about *A. suum* infection in humans. The migration of ascarid larvae through the human body can cause severe health problems such as eosinophilia, fever, coughing, enlarged liver, or pneumonia (Sakakibara *et al.*, 2002; Izumikawa *et al.*, 2011; Lamberton & Jourdan, 2015). In some cases, larvae can migrate to the brain or spinal cord causing neuro larva migrans (NLM) (Inatomi *et al.*, 1999; Umehara *et al.*, 2006) or eyes (Glickman *et al.*, 1987). It is called ascarid LMS in humans.

The transmission route of this infection varies in different countries, including either by accidental ingestion of egg embryonates in the soil or contaminated vegetables/drinking water (Matsuyama *et al.*, 1998; Tokojima *et al.*, 2004; Izumikawa *et al.*, 2011); or eating raw/undercooked meat and offals contaminated with the larval parasites (Choi *et al.*, 2012). The former methods are considered the main routes in American or Western European countries. By contrast, in East Asian countries such as Japan and South Korea, the later route has recently become more important due to eating habits. In Vietnam, the former routes could be considered as the main methods due to the habit of using pig manure for soil fertilizer in some locations (Vu *et al.*, 2007). However, the latter route has also become a risk because of the culture exchange amongst Asian countries, especially cuisine culture.

Chicken are one of the paratenic hosts of the ascarid roundworm because this roundworm does not develop into adult worms in chickens (Taira *et al.*, 2003; Azizi *et al.*, 2007; Yoshihara *et al.*, 2008). Laboratory experiments of *A. suum* infection in chickens have shown the pattern of ascarid larvae distribution. After hatching in the digestive system, ascarid larvae penetrated the intestinal wall, migrated to the liver, lungs, and eventually back to the liver (for *T. canis*) where they remained in the carcass (for *T. cati*) (Okoshi & Ushui, 1968; Taira *et al.*, 2003) or were eliminated from the chicken after 14 days (Yoshihara *et al.*, 2008). However, in natural infections, chickens that are reared freely outside in the environment (free-range chickens) could have a higher risk of getting ascarid eggs from the soil based on their temperament and become a paratenic host for them, especially if pig manure is used for fertilizer (Vu *et al.*, 2007). Because of this temperament, soil contaminated with ascarid eggs could be evaluated in addition to the detection of anti-ascarid antibodies in chickens (Campos de Silva *et al.*, 2015). Thus, the objectives of this study were to evaluate the contamination of chicken livers with *A. suum*, *T. canis*, and *T. cati* and then provide evidence of ascarid roundworm infections in humans through contact with contaminated soil or eating raw/undercooked chickens in Vietnam.

Materials and Methods

Parasites

A. suum, *Toxocara canis*, and *T. cati* adult worms were collected from naturally infected pigs, dogs, and cats, respectively, at local abattoirs around Hanoi. They were identified by morphology before performing the next steps. The roundworms were then thoroughly washed in saline solution and male worms were separated and kept in 70% alcohol for DNA extraction.

Liver samples

Ninety-four chickens were purchased from a fresh market in Trau Quy Town, Gia Lam district, Hanoi, Vietnam in order to collect the livers from naturally infected chickens. These free-range chickens were reared freely and derived from Hoa Binh, Bac Giang, and Hai

Duong provinces. The chickens then were necropsied to inspect the macropathology (white spot lesions) on the liver surfaces. Next, the livers were collected for the detection of *A. suum* by the nested multiplex PCR assay.

DNA extraction

The genomic DNA was extracted from adult *A. suum*, *T. canis*, and *T. cati* worms using a Blood- Animal- Plant preparation Kit (Jena Bioscience, Germany) following the manufacturer's instructions. DNA concentration was measured by NanoDrop 2000 (ThermoFisher Scientific, US) to detect the presence of DNA.

The alkaline-lysis method was employed for extracting gDNA from the liver tissue. Five hundred milligrams (500mg) of chicken liver was homogenized in a 15mL tube provided by BioMasher (Nippi, Tokyo, Japan), and 1.8 mL of 50mM NaOH was added. After boiling for 30 min, the samples were neutralized by 200µL of 1M Tris-HCl (pH 8.0). The mixture was vortexed thoroughly and centrifuged at 14,000×g for 10min. The supernatant was transferred to a new tube and stored at -20°C until analysis (Nguyen *et al.*, 2016).

Nested multiplex PCR assay

The nested multiplex PCR assay was applied to amplify a region of the ITS1 ribosomal RNA gene of *A. suum*, *T. canis*, and *T. cati*. The PCR reaction was performed in the total volume of 25 µl containing 1µL of template DNA and the

following PCR mixture: 12.5µL Master mix 2X (Phusa Biochem, Vietnam), 0.75µL of each primer at concentrations of 0.3mM (Phusa Biochem, Vietnam) and 10µL distilled water. The thermal cycling profile was 94°C for 2min, followed by 40 cycles with denaturation at 94°C for 15s, primer annealing at 52°C for 30s, and extending at 68°C for 1min, and a final elongation step at 72°C for 7min. The first PCR products were used as a template in the species-specific nested multiplex PCR, in which multiple primer sets were applied in the same tube with 0.75µL of each of species-specific forward and reverse at concentrations 0.3mM. The PCR conditions were similar to the first reaction, except for primer annealing was at 57°C (**Table 1**) (Wang *et al.*, 2018).

The amplification products were analyzed using 1.0% agarose gel electrophoresis. The amplification bands were visualized under UV light. A positive reaction was observed when the appearance of the specific sized bands of 208bp, 325bp, and 233bp corresponding with *T. canis*, *T. cati*, and *A. suum*, respectively (Wang *et al.*, 2018).

Results and Discussion

Macroscopic inspection on the liver surfaces

Most of the collected livers were normal in color and structure. However, 15 of the 94 chickens showed macropathological damage on their livers. White spot lesions ranging from

Table 1. Primer sequences that were used for the nested PCR and nested multiplex PCR (Wang *et al.*, 2018)

Primers		Sequences
Ascarid specific universal Primers (First PCR)	Ascarid	Forward: 5'-CGTCGGTAGCGATGAAAGGT-3' Reverse: 5'-TTAGTTTCTTTTCTCCGCT-3'
	<i>T. canis</i>	Forward: 5'-CTCGAGTCGACGAAGTATGTAC-3' Reverse: 5'-AATTGGGCCGCCATCATAC-3'
Species-specific primers (second PCR)	<i>T. cati</i>	Forward: 5'-GTAAGATCGTGGCAGCGTACGTA-3' Reverse: 5'-TCTTTGATGTCAAGACTTCAGCGC-3'
	<i>A. suum</i>	Forward: 5'-TCAACATTCATAGAGAATGGCATGT-3' Reverse: 5'-TACATCATTATTGTACGCTCATCT-3'



Figure 1. The white spot lesions on chicken livers infected naturally

1-10 in number and 0.1-0.5mm in diameter were observed on both surfaces of the livers (**Figure 1**). In addition, several different macropathological features were observed on the liver surfaces, such as damage caused by Marek's disease. However, those samples were excluded from this study.

The observation of white spot lesions on the liver surfaces was one piece of critical evidence of the migration of ascarid larvae through the chicken liver (Taira *et al.*, 2003; Azizi *et al.*, 2007; Yoshihara *et al.*, 2008). However, the white spot lesions were only recognized on day 7 in chickens with double or triple *A. suum* infections (Yoshihara *et al.*, 2008). White spot lesions were not observed if the chickens were not reinfected or in the cases of early infection (Yoshihara *et al.*, 2008).

In natural infection, chickens can ingest eggs more frequently, leading to a higher chance of larvae presenting in liver. Moreover, raw liver is also a special cuisine in some countries such as Japan and South Korea. This is one of the transmission routes by which humans get ascarid larva migrans syndrome (ascarid LMS) (Choi *et al.*, 2012). Thus, the liver samples were the target of selection for this study.

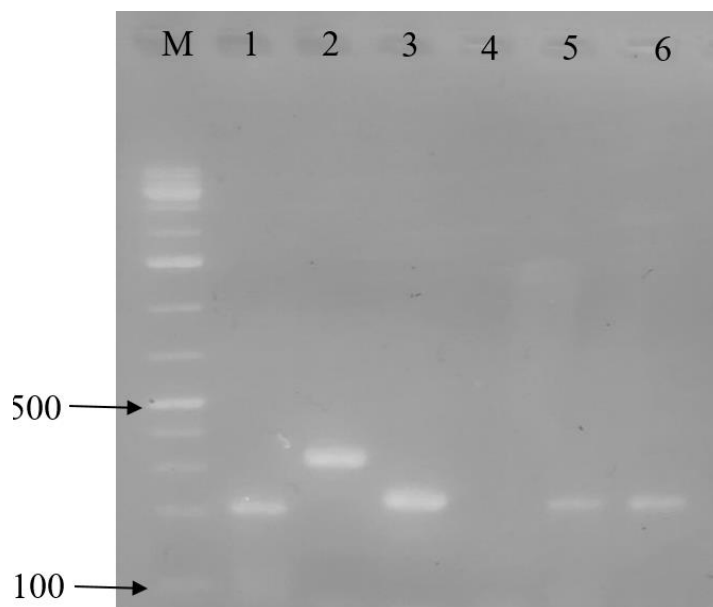
Detection of *A. suum* in the chicken livers

Before applying the nested multiplex PCR assay for the naturally infected liver samples,

positive control samples (ascarid adult-derived DNA) were successfully amplified. Of the 94 livers collected, two of them were positive and both of them showed amplification of a 233bp band, which is specific for *A. suum* (**Figure 2**). Meanwhile, *Toxocara* spp. DNA were not detected in this study.

The presence and existence of larvae in the liver depends on each agent. In the cases of *T. cati* and *A. suum*, the larvae exist in the liver around 14 days after the last experimental infection (Azizi *et al.*, 2007; Yoshihara *et al.*, 2008). For *T. canis*, after the larvae migrate to the lungs, they migrate back to the liver and exist there for ninety days (Okoshi & Usui, 1968; Taira *et al.*, 2003). The results of this study showed that the two positive samples of *A. suum* infection were not in livers showing white spot lesions. Thus, the detection of *A. suum* herein illustrated new infections of *A. suum* in those naturally infected chickens. Investigation of several free-range chicken farms showed that the farmers often plant fruit trees such as pomelo trees or litchi trees around and inside chicken-rearing areas in order to provide shade for the chickens, and they sometimes used pig manure to fertilize these trees without treatment.

Additionally, chickens were reared in old pigsties in some of the chicken farms (unpublished



Note: Lane M: 100 bp DNA ladder marker; Lanes 1-3: positive controls (lane 1: *T. canis* DNA; lane 2: *T. cati* DNA; lane 3: *A. suum* DNA); Lane 4: negative control; Lanes 5-6: *A. suum* DNA liver samples.

Figure 2. The results of *Ascaris suum* detection in naturally infected chickens

results from another study). Meanwhile, dogs or cats were usually raised outside with no/little chance of invading the chicken-rearing areas. Thus, it is possible that the chance of ingesting *A. suum* eggs was higher than with *Toxocara* eggs in free-range chickens. Moreover, *T. malaysiensis* was reported in cats in Hanoi (Le *et al.*, 2016). Thus, further studies need to be conducted to clarify this issue.

Conclusions

The detection of *A. suum* DNA in naturally infected chicken livers is a direct proof of soil contamination with *A. suum* eggs, but not a direct proof of the infectivity of larvae to a new host. However, the results of this study provided the evidence of chickens ingesting *A. suum* eggs via exposure to contaminated soil. Thus, people need to be made aware of the importance of treating pig manure before fertilizing plants, especially those with soil-related occupations. This paper also provides critical information on the potential evidence of *A. suum* infections in humans in Vietnam. Further studies need to be conducted to evaluate *A. suum* infections in humans in this country.

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References

- Azizi S., Oryan A., Sadjjadi S. M. & Zibaei M. (2007). Histopathologic changes and larval recovery of *Toxocara cati* in experimentally infected chickens. *Parasitology Research*. 102: 47-52.
- Beaver P. C., Snyder H., Carrera G. M., Dent J. H. & Lafferty J. W. (1952). Chronic eosinophilia due to visceral larvae migrans. *Pediatrics*. 9: 7-19.
- Campos-da-Silva D. R., da Paz J. S., Fortunato V. R., Beltrame, M. A., Valli L. C. & Pereira F. E. (2015). Natural infection of free-range chickens with the ascarid nematode *Toxocara* sp. *Parasitology Research*. 114(11): 4289-4293.
- Choi D., Lim J. H., Choi D. C., Lee K. S., Paik S. W., Kim S. H. & Huh S. (2012). Transmission of *Toxocara canis* via ingestion of raw cow liver: A cross-sectional study in healthy adults. *Korean Journal of Parasitology*. 50: 23-27.
- Chomel B. B., Kasten R., Adams C., Lambillotte D., Theis J., Goldsmith R., Koss J., Chioino C., Widjana D. P. & Sutisna P. (1993). Serosurvey of some major zoonotic infections in children and teenagers in Bali,

- Indonesia. Southeast Asian Journal of Tropical Medicine Public Health. 24: 321-326.
- Do T. D., Tran T. D., Nguyen T. H., Hoang Q. V., Do T. T. & Nguyen T. L. A. (2016). Current status of *Toxocara* spp. infection in humans in some study sites of Hanoi and Hung Yen, 2014-2015. *Journal of Malaria and Parasitic Disease Control*. 6(102): 10-16 (in Vietnamese).
- Hoenigl M., Valentine T., Zollner-schwetz I., Salzer H. J. F., Raggam R. B., Strenger V. & Krause R. (2010). Pulmonary ascariasis: Two cases in Austria ANS review of the literature. *Wiener klinische Wochenschrift*. 122: 94-96.
- Inatomi Y., Murakami T., Tokunaga M., Ishiwata K., Nawa Y. & Uchino M. (1999). Encephalopathy caused by visceral larva migrans due to *Ascaris* suum. *Journal of the Neurological Science*. 164: 195-196.
- Izumikawa K., Kohno Y., Izumikawa K., Hara H., Hayashi H., Maruyama H. & Kohno S. (2011). Eosinophilic pneumonia due to visceral larva migrans possibly caused by *Ascaris* suum: A case report and review of recent literatures. *Japanese Journal of Infectious Diseases*. 64: 428-432.
- Lamberton P. H. L. & Jourdan P. M. (2015). Human Ascariasis: Diagnostics update. *Current Tropical Medicine Reports*. 2: 189-200.
- Magnaval J. F., Michault A., Calon N. & Charlet J. P. (1994). Epidemiology of human toxocariasis in La Reunion. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 88: 531-533.
- Maruyama H., Nawa Y., Noda S., Mimori T. & Choi W. Y. (1996). An outbreak of visceral larva migrans due to *Ascaris* suum in Kyushu, Japan. *The Lancet*. 347: 1766-1767.
- Miller L. A., Colby K., Manning S. E., Hoenig D., McEvoy E., Montgomery S. & Sears S. (2015). Ascariasis in humans and pigs on small-scale farms, maine, USA, 2010-2013. *Emerging Infectious Diseases*. 21: 332-334.
- Le T. H., Nguyen T. L. A., Nguyen T. K., Nguyen T. B. N., Do T. T. T. & Gasser R. B. (2016). *Toxocara malaysiensis* infection in domestic cats in Vietnam – A emerging zoonotic issue? *Infection, Genetic and Evolution*. 37: 94-98.
- Nguyen Y. T. H., Wang Z. Z., Maruyama H., Horii Y., Nonaka N. & Yoshida A. (2016). Evaluation of real-time PCR assay for the detection of *Ascaris* suum contamination in meat and organ meats. *Journal of Food Safety*. 37(2): 1-6.
- Okoshi S. & Ushui M. (1968). Experimental Studies on *Toxascaris Leonina*. VI. Experimental Infection of Mice, Chicken and Earthworms with *Toxascaris leonina*, *Toxocara canis* and *Toxocara cati*. *Japanese Journal of Veterinary Medicine Science*. 30: 151-166.
- Pinelli E., Herremans T., Harms M. G., Hoek D. & Kortbeek L. M. (2011). *Toxocara* and *Ascaris* seropositivity among patients suspected of visceral and ocular larva migrans in the netherlands: Trends from 1998 to 2009. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*. 30: 873-879.
- Umehara F., Ookatsu H., Hayashi D., Uchida A., Douchi Y., Kawabata H. & Osame M. (2006). MRI studies of spinal visceral larva migrans syndrome. *Journal of Neurological Science*. 249: 7-12.
- Sakakibara A., Basa K., Niwa S., Yagi T., Wakayama H., Yoshida K. & Kimura E. (2002). Visceral larva migrans due to *Ascaris* suum which presented with eosinophilic pneumonia and multiple intra-hepatic lesions with severe eosinophil infiltration- outbreak in a Japanese area other than Kyushu. *Internal Medicine*. 41: 574-579.
- Taira K., Permin A. & Kapel C. M. O. (2003). Establishment and migration pattern of *Toxocara canis* larvae in chickens. *Parasitology Research*. 90: 521-523.
- Thompson D. E., Bundy D. A. P., Cooper E. S. & Schantz P. M. (1986). Epidemiological characteristics of *Toxocara canis* infection of children in a Caribbean community. *Bull WHO*. 64: 283-290.
- Tokojima M., Ashitani J. & Nakazato M. (2004). A case of eosinophilic pneumonia caused by visceral larva migrans due to *Ascaris* suum. *The Japanese Association for Infectious Diseases*. 78: 1036-1040.
- Tran T. D. (2014). Actual situation study, some factors has risk to infect larva of *Toxocara canis* in human and effect of treatment by Albendazole at 2 commune of An Nhon District, Binh Dinh Province (2011-2012). PhD thesis (in Vietnamese).
- Vu T. K. V., Tran M. T. & Dang T. T. S. (2007). A survey of manure management on pig farms in Northern Vietnam. *Livestock Science*. 112: 288-297.
- Wang Z. Z., Shibata M., Nguyen Y. T. H., Hayata Y., Nonaka N., Maruyama H. & Yoshida A. (2018). Development of nested multiplex polymerase chain reaction (PCR) assay for the detection of *Toxocara canis*, *Toxocara cati* and *Ascaris* suum contamination in meat and organ meats. *Parasitology International*. 67: 622-628.
- Yoshihara S., Hattori J., Nishizono K., Kawamura A., Shimozaki K., Nishida Y. & Hirayama N. (2008). Hepatic lesions caused by migrating larvae of *Ascaris* suum in chickens. *Journal of Veterinary Medicine Science*. 70: 1129-1131.